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Interleukin-10-secreting type 1 regulatory T cells in rodents and humans

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Summary: Interleukin-10 (IL-10)-secreting T regulatory type 1 (Tr1) cells are defined by their specific cytokine production profile, which includes the secretion of high levels of IL-10 and transforming growth factor-β (TGF-β), and by their ability to suppress antigen-specific effector T-cell responses via a cytokine-dependent mechanism. In contrast to the naturally occurring $CD4^+CD25^+$ T regulatory cells (Tregs) that emerge directly from the thymus, Tr1 cells are induced by antigen stimulation via an IL-10-dependent process *in vitro* and *in vivo*. Specialized IL-10-producing dendritic cells, such as those in an immature state or those modulated by tolerogenic stimuli, play a key role in this process. We propose to use the term Tr1 cells for all IL-10-producing T-cell populations that are induced by IL-10 and have regulatory activity. The full biological characterization of Tr1 cells has been hampered by the difficulty in generating these cells *in vitro* and by the lack of specific marker molecules. However, it is clear that Tr1 cells play a key role in regulating adaptive immune responses both in mice and in humans. Further work to delineate the specific molecular signature of Tr1 cells, to determine their relationship with $CD4^+CD25^+$ Tregs, and to elucidate their respective role in maintaining peripheral tolerance is crucial to advance our knowledge on this Treg subset. Furthermore, results from clinical protocols using Tr1 cells to modulate immune responses *in vivo* in autoimmunity, transplantation, and chronic inflammatory diseases will undoubtedly prove the biological relevance of these cells in immunotolerance.

Keywords: *interleukin-10, regulatory T cells, Tr1 cells*

Introduction

T regulatory cells (Tregs) are crucial players in the induction of peripheral tolerance to self and foreign antigens. A large body of evidence attributes an important role to these cells in the immunological dysregulation underlying autoimmune diseases, chronic inflammatory diseases, and cancer, as well as in the immunobiology of transplantation. The two most relevant classes of Tregs described within the $CD4^+$ subset are T regulatory type 1 (Tr1) cells (1, 2) and $CD4^+CD25^+$ Tregs (3, 4). These two Treg subsets differ in a number of important biological features, including their specific cytokine secretion profile, cellular markers, ability to differentiate in response

to antigen-specific stimuli, and dependency on cytokines vs. cell–cell contact mechanisms for mediating suppressive activity (4, 5).

Interleukin-10 (IL-10) was first discovered based on its capacity to inhibit cytokine secretion by mouse T-helper 1 (Th1) cells (6, 7), and this cytokine is an essential molecule in the mechanism underlying suppression mediated by Tregs. Interleukin-10 has anti-inflammatory and suppressive effects on most hematopoietic cells, and it indirectly suppresses cytokine production and proliferation of antigen-specific $CD4^+$ T effector cells, by inhibiting the antigen-presenting capacity of different types of professional antigen-presenting cells (APCs), including dendritic cells (DCs), Langerhan's cells, and macrophages. In contrast, IL-10 has stimulatory effects on effector $CD8^+$ T cells by enhancing their cytotoxic capacity and proliferation (8–11). Interleukin-10 also has an anti-apoptotic effect on B cells, is involved in B-cell isotype switching, and plays a role in autoimmune diseases with underlying B-cell dysregulation such as systemic lupus erythematosus (12, 13). Importantly, IL-10 is produced by subsets of $CD4^+$ T cells with regulatory functions.

The first type of IL-10-producing Tregs described at the clonal level was designated Tr1 cells (14). These cells are induced by an IL-10-dependent process *in vitro* and *in vivo*. They are defined by their capacity to produce high levels of IL-10 and transforming growth factor-β (TGF-β). These cytokines play a key role in suppression of antigen-specific effector T-cell responses by both murine and human Tr1 cells. Tr1 cells can be distinguished from Th1 and Th2 cells, because they produce low amounts of IL-2 and no IL-4. Depending on the experimental conditions used for their induction, Tr1 cells can also produce IL-5 and interferon-γ (IFN-γ), although the latter cytokine is produced at levels that are at least one log lower than Th1 cells (15).

After the first report on the characterization of Tr1 cells (14), IL-10-producing T cells with regulatory functions have been generated using a variety of experimental conditions (16–25). These Tregs have not always been defined as Tr1 cells, because their cytokine expression patterns differed slightly from the canonical profile initially described for Tr1 cells [$IL\text{-}10^+IL\text{-}5^+IL\text{-}4^-IL\text{-}2^{+/-}IFN\text{-}\gamma^+$ (14, 15, 26)]. In many instances, these deviations can be explained by the different experimental conditions used for the induction of Tregs. We propose here that the term Tr1 cells should be used for all IL-10-secreting T-cell populations with regulatory activity, the induction of which is IL-10 dependent.

In the past 10 years, the functional role of Tr1 cells has been demonstrated in different T-cell-mediated diseases and in the setting of allogeneic transplantation. Nonetheless, the precise molecular mechanisms involved in the differentiation and function of these cells remain largely elusive. This review describes the current state of knowledge regarding the biological features and different stimuli that induce Tr1 cells *in vitro* or *in vivo*. The biological relationship between Tr1 and $CD4^+CD25^+$ Tregs and the role of these two $CD4^+$ Treg subsets in primary immunodeficiencies, autoimmunity, infectious diseases, and cancer is reviewed. Finally, the potential use of Tregs in cellular therapy after transplantation of allogeneic hematopoietic stem cells (HSCs) is described.

Biological features of Tr1 cells

Tr1 cells can be generated *in vitro* and *in vivo* upon priming of naïve T cells with antigen in the presence of IL-10. These IL-10-producing $CD4^+$ Tr1 cells are characterized by a unique pattern of cytokine production, which is distinct from that of classical Th1 and Th2 cells (14). Upon T-cell receptor (TCR)-mediated activation, Tr1 cells produce high levels of IL-10 and TGF-β and IL-5, low amounts of IFN-γ and IL-2, and no IL-4. Interleukin-10 secreted by Tr1 cells is detectable as soon as 4 h after activation. Thereafter, the levels of IL-10 increase rapidly and the highest concentration is reached 12–24 h after activation (26). Different populations of IL-10-producing T cells with regulatory function have been described [reviewed Tregs (16)] secreting IL-10 but not other cytokines can be induced by immunosuppressive drugs (23, 25), soluble protein, and peptide antigens (18, 19, 21, 22, 28). It has also been proposed that Tr1 cells can be generated from fully differentiated Th1 and Th2 cells upon chronic stimulation, maintaining only their ability to secrete IL-10 but not other cytokines (29). Depending on the experimental systems used for the induction of Tr1 cells, their cytokine profile can vary with regards to TGF-β, IFN-γ, and/or IL-5 production, but their levels of IL-10 production, which represents the true hallmark of Tr1 cells, are invariably high. Interleukin-4 production is consistently undetectable.

$CD8^+$ Tr1-like cells, which produce IL-10, have also been described (30, 31). These cells are anergic, suppress proliferative responses via IL-10, and can be generated *in vitro* by stimulation of naïve $CD8^+$ T cells with either activated plasmacytoid DCs (30) or IL-10-modulated myeloid DCs (31). In these studies, induction of $CD8^+$ Tr1-like cells was shown to be IL-10 dependent. We recently demonstrated that a new monoclonal antibodies, which recognizes the RO and RB isoforms of CD45, also induces anergic antigen-specific $CD8^+$ Tr1-like cells with suppressive activity (32).

Little is known about the downstream signaling events that lead to Tr1 cell activation. Upon polyclonal TCR engagement, Tr1 cell clones display normal calcium flux and tyrosine phosphorylation (M. K. Levings, unpublished data). Recently, it has been demonstrated that anergic murine IL-10-producing Tregs display an intact signaling pathway downstream of IL-10 and IL-2. Preserved responses to these cytokines are important for maintaining antigen-specific regulatory functions mediated by IL-10 as well as effector functions mediated by IL-2 (33).

Cytokine and chemokine receptors expressed by Tr1 cells

Tr1 cells have a low proliferative capacity but can expand in the presence of IL-2 and IL-15, independently from their activation via the TCR (34). This finding is consistent with the observation that activated Tr1 cells express the IL-2 receptor α chain (IL-2Rα) and high levels of the IL-15Rα chain, together with both the IL-2/IL-15Rβ and IL-2/IL-15Rγ chains. Following TCR-mediated activation, Tr1 cells express normal levels of activation markers such as CD40L, CD69, CD28, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and human leukocyte antigen-DR (HLA-DR) (34). Human Tr1 cells in the resting phase express both Th1-associated (CXCR3 and CCR5) and Th2-associated (CCR3, CCR4, and CCR8) chemokine receptors (35). Interestingly, CCR8 is expressed at higher levels compared with Th2 cells, and upon activation, human Tr1 cells migrate preferentially in response to I-309, a ligand for CCR8 (35). CCR8 seems also to be relevant *in vivo*, because it has been recently demonstrated that in a model of helminth infection, its expression is strongly associated with IL-10-producing $CD4^+$ T cells, which resemble Tr1 cells (36). In contrast, $CD4^+$ T cells from normal donors with a Tr1 cell phenotype have been found to express CCR9, suggesting that these cells home to the gut (37). Although the *in vivo* migratory capacity of Tr1 cells has not been analyzed in detail, it has been suggested that Tr1 cells display an enhanced and selective capacity to migrate to inflamed tissues during an active immune response (38). However, upregulation of the chemokine receptors known to be involved in the specific migration to inflammatory sites was not observed on Tr1 cells (38).

Tr1 cell markers

Significant efforts to identify specific markers expressed by Tr1 cells to allow their discrimination from the naturally occurring $CD4^+CD25^+$ Tregs have been made. Naturally occurring $CD4^+CD25^+$ Tregs constitutively express the transcription factor forkhead box protein 3 (Foxp3), which controls their differentiation (39) and is considered a specific marker for these Tregs. Several investigators have demonstrated that Tr1 cells do not constitutively express Foxp3, but upon activation, it can be upregulated to levels similar to those observed in activated $CD4^+CD25^-$ T cells (25, 40, and R. Bacchetta, unpublished data). Currently, no specific markers for Tr1 cells have been identified. However, comparative analysis of gene expression profiles of murine Tr1 and $CD4^+CD25^+$ Tregs revealed selective expression of the repressor of GATA-3 (ROG) in the former but not in the latter cell population (41). It has to be noted that ROG is not specific for Tr1 cells, since it is also expressed in activated Th cells.

IL-15 is a Tr1 cell growth factor

Tr1 cells are anergic and proliferate poorly upon polyclonal TCR-mediated or antigen-specific activation (14, 26), and these cells do not expand significantly under standard culture conditions. The autocrine production of IL-10 by Tr1 cells contributes to their low proliferative capacity, since addition of anti-IL10 monoclonal antibody partially restores this response (14, 26). We demonstrated that IL-15 supports Tr1 cell proliferation, even in the absence of TCR activation (34), and in combination with IL-2, significant expansion of Tr1 cell clones is obtained *in vitro* (34). Long-term culture in IL-15 does not alter the phenotype or function of Tr1 cell clones, although it does enhance production of IFN-γ. Thus, IL-15 is an important growth factor for Tr1 cell clones *in vitro* and may represent a survival factor for Tr1 cells *in vivo*. This notion is consistent with the observation that IL-15 sustains survival of memory T cells, which constitutively express IL-2/IL-15Rβ chain (42, 43).

Mechanisms of suppression

Tr1 cells regulate immune responses through the secretion of the immunosuppressive cytokines IL-10 and TGF-β, and they suppress both naïve and memory T-cell responses *in vivo* and *in vitro* (14, 23, 26, 27, 44) (*Tables 1 and 2*). Although antigen-specific Tr1 cells need to be activated via the TCR in order to exert their suppressive function, once activated, Tr1 cells can mediate bystander suppressive activity against other antigens. This bystander suppression is likely mediated by the local release of IL-10 and TGF-β (38), which act on both APCs and T cells. IL-10 downregulates expression of costimulatory molecules and pro-inflammatory cytokine production by APCs and directly inhibits IL-2 and tumor necrosis factor-α (TNF-α) production by $CD4^+$ T cells (45). Similarly, TGF-β downregulates the function of APCs (46) and inhibits proliferation and cytokine production by T cells (47). The suppressive effects of Tr1 cells are typically either partially or fully

Table 1. Murine antigen-specific T regulatory type 1 cells

Antigen specificity	System	Mode of induction/isolation	Modulated pathology	Reference
Self-antigens				
Anti-islet	*in vivo*	Tetramers	Diabetes	(233, 234)
GAD peptides	*in vivo*	Immunization	Diabetes	(235)
GAD peptides	*in vitro*	Immunization	Diabetes	(236)
MBP, PLP peptides	*in vivo*	Immunization	EAE	(237)
MOG peptides	*in vivo*	MOG intranasally	MCAO	(238)
Alloantigens	*in vivo*	IL-10 + Rapamycin	Islet Tx	(148)
Mycobacterium vaccae	*in vivo*	*Mycobacterium vaccae*	Airway inflammation	(176, 239)
FHA	*in vivo*	*Bordetella* infection	*Bordetella* infection	(116)
Cholera toxin	*in vivo*	Cholera toxin	Infection	(177, 178)
Helicobacter hepaticus	*in vivo*	*Helicobacter hepaticus*	Colitis	(181)
OVA	*in vitro*	High IL-10	IBD	(14, 86)
OVA	*in vitro*	Vit D3+ Dexa	EAE	(23)
OVA	*in vitro*	CD45RB DC	IBD	(146)
OVA	*in vivo*	OVA intranasally	Airway inflammation	(18, 19)
OVA	*in vivo*	CD8α+ DC	Airway inflammation	(240)

Dexa, dexamethasone; EAE, experimental allergic encephalomyelitis; GAD, glutamic acid decarboxylase; IBD, inflammatory bowel disease; IL-10, interleukin-10; MBP, myelin basic protein; MCAO, middle cerebral artery occlusion; MOG, myelin oligodendrocyte glycoprotein; OVA, ovalbumin; PHA, filamentous hemagglutinin; PLP, proteolipid apoprotein; Tx, transplantation; Vit D3, vitamin D3.

Table 2. Human antigen-specific T regulatory type 1 cells

Antigen specificity	System	Mode of induction/isolation	Modulated pathology	Reference
Self-antigens				
Self-HLA	*in vivo*	?	?	(159)
RhD	*in vivo*	?	Autoimmune hemolytic anemia	(162)
Desmoglein-3	*in vivo*	?	*Pemphigus vulgaris* ?	(44)
Heavy chain ferritin	*in vitro*	DC modulation	?	(122)
HLA-DR1	*in vitro*	CD2 costimulation	?	(56)
Alloantigens				
Allo-HLA	*in vivo*	?	GVHD	(26, 155)
Allo-HLA	*in vivo*	?	Kidney or liver Tx	(158)
Allo-HLA	*in vitro*	Immature DCs	GVHD/Tx	(24, 40)
Non-harmful antigens				
Gliadin	*in vitro*	High IL-10	Celiac disease	(C. Gianfrani, manuscript in preparation)
Infectious antigens				
Lactobacillus reuteri, L. casei	*in vitro*	DC	Infection ?	(117)
Tetanus toxoid	*in vitro*	High IL-10	Infection ?	(34)
Mycobacterium tubercolosis	*in vivo*	*Mycobacterium tubercolosis*	Infection	(20)
Helminth	*in vivo*	Helminth	Infection	(241)
HCV	*in vivo*	HCV	Infection	(179)
EBV LMP1	*in vivo*	EBV	Infection	(185)
ALP of *Plasmodium falciparium*	*in vivo*	*Plasmodium falciparium*	Infection	(242)
Allergens				
Nickel	*in vivo*	?	Allergy	(48)
Insect venom	*in vivo*	?	Allergy	(164)
Cat allergen	*in vivo*	?	Allergy	(165)
DNCB	*in vivo*	?	Allergy	(243)
Der p 1, Bet1	*in vivo*	?	Allergy	(166, 244)
Phleum pratense	*in vivo*	Grass pollen therapy	Allergy	(245)
Ferruginol	*in vitro*	DC	Allergy	(118)
Tumor antigens				
Myeloma antigen	*in vitro*	DC	Cancer	(119)
Cyclooxygenase-2	*in vitro*	DC	Cancer	(120)

APL, altered peptide ligand; Bet 1, *Betula verrucosa*; DC, dendritic cell; Der p 1, *Dermatophagoides pteroynyssinus* 1; DNCB, 2,4 dinitrochlorobenzene; EBV, Epstein–Barr virus; GVHD, graft-vs-host disease; HCV, hepatitis C virus; IL-10, interleukin-10; LMP1, latent membrane protein 1; Tx, transplantation.

reversed by addition of anti-IL10 and anti-TGF-β neutralizing monoclonal antibodies (5, 14, 26, 40, 48), but additional mechanisms may also contribute. Interestingly, Tr1 cells generated *in vitro* by crosslinking of CD3 and CD46, a complement regulatory protein, suppress T-cell responses via an IL-10 and granzyme B/perforin-dependent mechanism (49–51). IL-10-producing Tregs that do not mediate suppression of effector T cells via IL-10 have also been described (24, 25). It cannot be excluded that some of these IL-10-producing Tregs represent Tr1 cells arrested during the initial steps of differentiation, when they are already anergic and suppressive but their suppressive activity is not yet dependent on IL-10. It is possible that under the appropriate conditions these cells would fully differentiate into *bona fide* Tr1 cells mediating suppression through IL-10. Therefore, we propose that these IL-10-producing Tregs, which require IL-10 for their induction, are termed Tr1cells.

Relationship between Tr1 cells and $CD4^+CD25^+$ Tregs and the role of Foxp3

Tr1 cells represent a unique subset of $CD4^+$ Tregs, which is distinct from the extensively characterized $CD4^+CD25^+$ Treg subset (reviewed in 5). Tr1 cells are inducible from naïve cells *in vitro* and *in vivo* and therefore can be considered part of the adaptive immune response. On the contrary, natural $CD4^+CD25^+$ Tregs are selected in the thymus and thus have predefined antigen specificity (52, 53). $CD4^+CD25^+$ Tregs are more easily identified on the basis of cell surface markers and expression of Foxp3. More recently, many groups have found that $CD4^+CD25^+$ Tregs can also be induced in the periphery from naïve precursors (54, 55). Currently, there are no known phenotypic or functional differences between the natural and these so-called antigen-induced $CD4^+CD25^+$ Tregs.

Like Tr1 cells, $CD4^+CD25^+$ Tregs have a key role in controlling peripheral tolerance to a variety of antigens. Initially, they were thought to primarily control responses to self-antigens, based on the finding that depletion in neonatal mice results in systemic autoimmunity (56). Further research in mouse models and human studies, however, has now firmly established that $CD4^+CD25^+$ Tregs control responses not only to self-antigens but also to allergens, tumor antigens, transplanted foreign tissue, and infectious organisms (5, 52). Their detailed phenotype and biological function is discussed in other articles in this volume of *Immunological Reviews*.

We and others have shown that *in vitro* induction of Tr1 cells occurs independently from $CD4^+CD25^+$ Tregs and that Tr1 cells do not express high levels of CD25 or Foxp3, a transcription factor associated with the development of $CD4^+CD25^+$ Tregs (5, 25, 40, 57). In addition, clonal analysis of human $CD4^+CD25^+$ Tregs did not reveal the presence of Tr1 cells within the $CD25^{bright}$ pool, providing further evidence that they represent distinct populations (58).

Role of Foxp3 in murine $CD4^+CD25^+$ Tregs

The most important recent advance in our understanding of $CD4^+CD25^+$ Tregs came from the study of a naturally occurring mouse mutant (the scurfy mouse), which suffers from multi-organ autoimmunity due to uncontrolled $CD4^+$ T-cell proliferation. Recently, a two base-pair insertion in the Foxp3 transcription factor, which leads to a frameshift mutation, was identified as the mutant gene responsible for the phenotype (59). Subsequent studies demonstrated that scurfy mice completely lack $CD4^+CD25^+$ Tregs and that adoptive transfer of this missing population can correct the autoimmune phenotype (60, 61). In addition, it was found that $CD4^+CD25^+$ Tregs express high levels of Foxp3 (62). More recent studies in mouse models have focused on analysis of Foxp3-transgenic mice (63) and ectopic expression of Foxp3 in naïve $CD4^+$ T cells (62). It has been demonstrated that ectopic expression of Foxp3 can endow murine T cells with regulatory capacities *in vitro* and *in vivo* (64). From these studies, it can be concluded that, at least in murine cells, expression of Foxp3 appears to be necessary and possibly also sufficient for the development of $CD4^+CD25^+$ Tregs.

Role of Foxp3 in human $CD4^+CD25^+$ Tregs

It is now well-established that human natural $CD4^+$ $CD25^+$ Tregs express high levels of Foxp3 constitutively (65). However, in contrast to results described in murine cells, in humans it is still controversial whether Foxp3 overexpression in naïve $CD4^+$ T cells is sufficient to confer a regulatory function (66, 67). Our studies demonstrated that retroviral mediated ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells does not result in a strong and stable suppressive capacity equivalent to that of natural $CD4^+CD25^+$ Tregs (67), whereas Sakaguchi and coworkers (66) reported high suppressive activity after Foxp3 gene transfer. Of note is that in human cells, Foxp3 is also expressed by activated non-suppressive $CD4^+CD25^-$ T cells (67), suggesting that in addition to this transcription factor other components may be required for optimal suppressor activity (68). Moreover, human cells co-express an alternatively spliced isoform of Foxp3, which lacks exon 2. Although the precise function of these two isoforms remains to be fully defined, preliminary data suggest that their

biological roles may not be equivalent (67, 68). Until the precise molecular function of Foxp3 is established, caution should be exercised when extrapolating data from murine models to humans.

Peripheral generation of $CD4^+CD25^+$ Tregs

Much effort has been placed on defining whether $CD4^+CD25^+$ Tregs arise exclusively in the thymus or whether antigen-specific cells can also differentiate *de novo* in the periphery. In humans, peripheral development of $CD4^+CD25^+$ Tregs appears to be a stochastic process, with a certain fraction of activated cells remaining $CD25^{bright}$, beginning to express Foxp3, and acquiring suppressive capacity (54, 55). Other studies suggest that agents such as TGF-β can enhance this process (69, 70). Of note is a recent study that elegantly demonstrates that a significant proportion of $Foxp3^+$ cells are contained within the $CD25^-$ fraction and that if these cells are depleted, *de novo* expression of Foxp3 cannot be induced *in vitro* or *in vivo* (71). It is therefore possible that 'induction' of $CD4^+CD25^+$ Tregs from $CD25^-$ T cells actually represents preferential expansion of a pre-existing subset.

Role of IL-10 and TGF-β in suppression by $CD4^+CD25^+$ Tregs

It is often difficult to attribute suppressive activity *in vivo* to Tr1 cells or $CD4^+CD25^+$ Tregs. Of particular interest is whether $CD4^+CD25^+$ Tregs have the capacity to produce IL-10 and/or TGF-β and to mediate suppression via cytokines. We and others found that IL-10 has no role in the function of human $CD4^+CD25^+$ Tregs *in vitro* (27, 72, 73). In contrast, in some mouse models, IL-10 does seem to play a role (74). Although we and others observed a small but consistent role for TGF-β in suppression by human $CD4^+CD25^+$ T-cell clones, neutralizing antibodies are never able to completely reverse suppression *in vitro* (58, 72, 73, 75, 76). In contrast, some murine models, particularly those analyzing mucosal immune responses, suggest that there is a role for cell-surface or secreted TGFβ in $CD4^+CD25^+$ Treg-mediated suppression (77–80). However, other studies have clearly ruled out a functional role for this cytokine in Treg-mediated suppression *in vitro* and *in vivo* (81–83). A recent report, which suggests that TGF-β is important for maintaining expression of Foxp3 and thus the suppressive function of $CD4^+CD25^+$ Tregs, but it is not directly involved in their mechanism of action, may reconcile these discrepancies (84).

These conflicting findings with respect to the role of cytokines in the suppressive function of $CD4^+CD25^+$ Tregs also may be due to the fact that these cells can be induced to produce immunoregulatory cytokines under some conditions or in response to certain pathogens, and in these specialized cases, IL-10 and/or TGF-β are required for suppression. An alternative but not mutually exclusive possibility is that subsets of $CD4^+CD25^+$ Tregs may have a role in inducing IL-10 and/or TGF-β-producing Tr1 cells (72, 85). *In vivo* antibody-based neutralization studies would obviously not be able to discriminate between these possibilities.

Networks of Tr1 and $CD4^+CD25^+$ Tregs

It can be concluded that although these two subsets of Tregs have non-redundant roles in peripheral tolerance, they probably achieve their effects in synergy in interdependent networks of cytokine-dependent (i.e. Tr1) and -independent (i.e. $CD4^+CD25^+$ Treg) regulation (*Fig. 1*). The relative importance of Tr1 vs. $CD4^+CD25^+$ Tregs, in any given instance, is likely dictated by the nature of the antigen, the context of antigen presentation, and the biology of specific tissues. One further level of control could be trafficking, as Tr1 and $CD4^+CD25^+$ Tregs appear to have distinct migratory behavior (86, 87). Finally, the time at which these two different Treg subsets

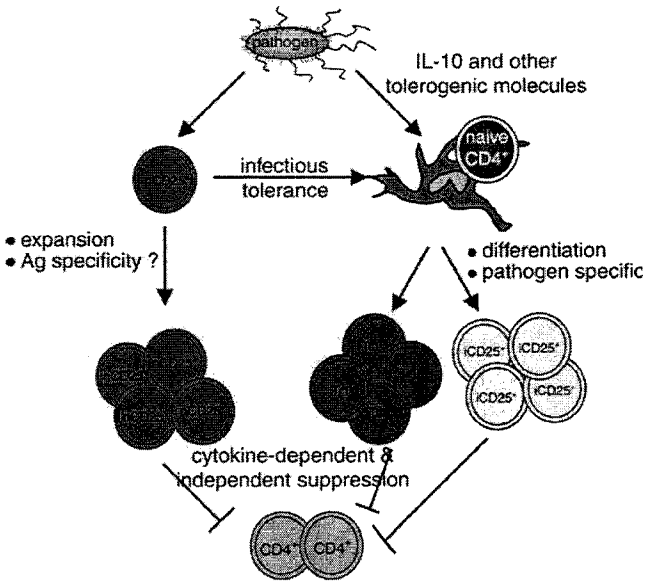

Fig. 1. Networks of $CD4^+$ regulatory T cells (Tregs) control immune responses to pathogens. Pathogens have evolved sophisticated mechanisms to promote the development of different subsets of Tregs. Early in the immune response, they directly promote the expansion of naturally occurring (n) $CD4^+CD25^+$ Tregs. In parallel, they secrete cytokines and express a variety of tolerogenic molecules which modulate dendritic cells (DCs) and/or T cells. T regulatory type 1 cells and antigen-induced (i) $CD4^+CD25^+$ Tregs arise upon priming by tolerized DCs, and together with $nCD4^+CD25^+$ Tregs, they form an immunoregulatory network that acts on effector T cells. Unresolved questions relate to the antigen specificity of the different subsets and their relative contributions to the overall response.

play a role in the modulation of the immune response may also be different. It is clear that natural $CD4^+CD25^+$ Tregs can be recruited and activated early during an immune response to control its magnitude, whereas adaptive Tr1 cells, which are induced upon repeated antigen stimulation, may act later to dampen the immune response and to restore and maintain tolerance.

In vitro expansion of $CD4^+CD25^+Foxp3^+$ Tregs

$CD4^+CD25^+Foxp3^+$ Tregs are a source of Tregs with the potential to be used for cellular therapy, but this application is currently hampered by their limited availability and poor capacity to be expanded *in vitro*. Significant efforts have been made to develop methods to expand this Treg subset while preserving its regulatory phenotype. Polyclonal activation in the presence of high levels of IL-2 can stimulate antigen-non-specific proliferation of $CD4^+CD25^+Foxp3^+$ Tregs isolated from both mice and humans (88–90). Importantly, *in vitro*-expanded $CD4^+CD25^+$ Tregs maintain expression of several markers including CD25, CTLA-4, CD62L, glucocorticoid-induced TNF receptor (GITR), and Foxp3, and preserve their regulatory function (58, 76, 90). Moreover, *in vitro*-expanded $CD4^+CD25^+$ Tregs are functional *in vivo*. They can protect mice from lethal graft-vs.-host disease (GVHD) in a preclinical animal model of bone marrow transplantation (89). The major limitation of using polyclonally amplified $CD4^+CD25^+$ Tregs resides in the risk of co-expanding effector T cells. Indeed, we have shown that in humans, even the brightest $CD25^+$ T cells contain approximately 50% of recently activated T cells, which ultimately overgrow the Tregs upon prolonged culture (58, 76). We recently reported an alternative method to selectively enrich for $CD4^+CD25^+Foxp3^+$ Tregs *in vitro* by repetitive activation of murine $CD4^+$ T cells in the presence of rapamycin. This drug blocks the expansion of murine effector T cells, while sparing the $CD4^+CD25^+$ Tregs (91). Consequently, rapamycin-treated T cells are enriched in $CD25^{bright}Foxp3^+$ Tregs, which are suppressive both *in vitro* and after adoptive transfer *in vivo*. Addition of rapamycin to these cultures may therefore represent an important method to selectively expand $CD4^+CD25^{bright}Foxp3^+$ Tregs for cellular therapy.

Differentiation and expansion of Tr1 cells *in vitro*

Methods to differentiate Tr1 cells in the absence of DCs

Tr1 cells arise from naïve precursors in specialized environments. Interleukin-10 is considered to be the driving force in Tr1 cell generation, based on experiments in which antigen-specific Tr1 cells were induced *in vitro* by repeated TCR stimulation in the presence of high doses of IL-10 (14). Activation in the presence of vitamin D3 and dexamethasone can also induce Tr1 cell differentiation via stimulation of autocrine IL-10 production (23, 25). Furthermore, treatment of mixed lymphocyte reaction (MLR) cultures with IL-10 (92) (*Fig. 2A*), and TGF-β in the mouse, results in anergy. These alloreactive anergic $CD4^+$ T cells are incapable of inducing GVHD and suppress responses of naïve T cells, after *in vivo* transfer into histo-incompatible recipients (53–55). Alloreactive Tr1 cell clones from healthy individuals were originally isolated by limiting dilution using *in vitro* IL-10-anergized $CD4^+$ T cells (14).

Although IL-10 is necessary, it is probably not sufficient for the full differentiation of Tr1 cells (27). We demonstrated, in an *in vitro* system using artificial APCs expressing high levels of CD58 and CD80, that addition of exogenous IL-10 results in a relatively small increase in IL-10-producing Tr1 cells. Co-addition of IFN-α, which can further promote autocrine IL-10 production, results in efficient differentiation of human $CD4^+$ Tr1 cells *in vitro* (27). Signaling via CD2, the ligand for CD58, can also enhance the induction of Tr1 cells (96). Atkinson and colleagues (49) identified co-signaling via CD46 as another physiological inducer of Tr1 cells. However, it is still unclear whether these CD3/CD46-stimulated T cells are *bona fide* Tr1 cells or if they represent a distinct inducible Treg subset. We recently reported that an anti-human CD45RO/RB monoclonal antibody is a potent immunomodulant that induces antigen-specific anergic T cells, which display a Tr1 phenotype and suppress IFN-γ production and proliferation of effector T cells via IL-10 and TGF-β (32). Further defining the stimuli, in addition to IL-10, that drive the differentiation of homogenous populations of antigen-specific Tr1 cells will significantly enhance our understanding of their biology and their potential for clinical use.

Induction of Tr1 cells by immature DCs

Dendritic cells are professional APCs that classically initiate antigen-specific immune responses upon infection (97). This process involves the terminal maturation of DCs, typically induced by agents associated with microbial infection. In contrast, in the steady state, DCs remain immature (iDCs), and growing evidence indicates that in this form they induce tolerance via deletion of antigen-specific effector T cells and/or differentiation of Tregs (2, 24, 98–103). For example, repetitive stimulation of naïve cord blood $CD4^+$ T cells with allogeneic iDCs results in the differentiation of IL-10-producing Tregs (24) that suppress T-cell responses via a cell-contact

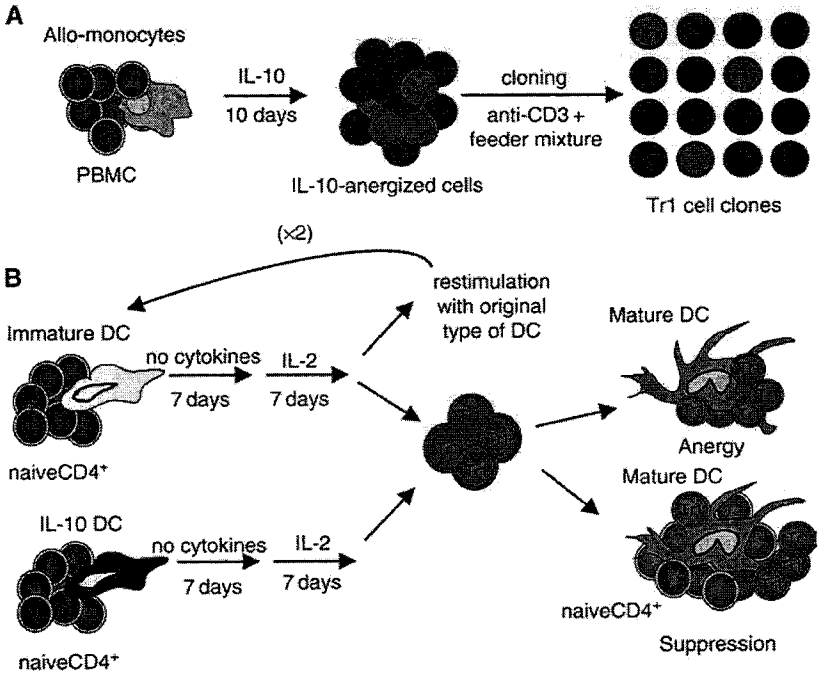

Fig. 2. In vitro differentiation of T regulatory type 1 (Tr1) cell clones and lines using monocytes or dendritic cells (DCs). (A) Peripheral blood mononuclear cells are stimulated *in vitro* with irradiated allogeneic monocytes in the presence of recombinant human interleukin-10 (IL-10) (i.e. primary mixed lymphocyte reaction). To demonstrate that the IL-10-anergized cells are highly enriched in Tr1 cells, cultures are cloned in the presence of a feeder mixture consisting of allogenic PBMC and EBV-LCL and repetitively stimulated through the T-cell receptor. The anergic cells acquire a unique cytokine production profile (i.e. IL-10$^+$TGF-β$^+$IL-4$^-$IL-2$^{low/-}$) that is a hallmark of Tr1 cells. (B) Peripheral blood CD4$^+$CD45RO$^-$ T cells are stimulated with allogeneic immature DCs (iDCs) at 10:1 ratio for 7 days and expanded for an additional 7 days in the presence of low amounts of recombinant human IL-2. Subsequently, T-cell lines are restimulated twice with DCs from the original donor for 7 days. Alternatively, peripheral blood CD4$^+$CD45RO$^-$ T cells can be stimulated once with IL-10 DCs at 10:1 ratio for 7 days and expanded for an additional 7 days in the presence of low amounts of recombinant human IL-2. The resulting Tr1 cell lines are anergic in response to mature allogeneic DCs (mDCs) and suppress responses of autologous CD4$^+$ T cells activated with mDCs.

dependent mechanism. Furthermore, we recently reported that repeated stimulation of naïve peripheral blood CD4$^+$ T cells with allogeneic iDCs induces the differentiation of human Tr1 cells *in vitro* (40) (Fig. 2B). In this system, T cells become increasingly hyporesponsive to re-activation with mature DCs, and after three rounds of stimulation with iDCs, they are profoundly anergic and acquire regulatory function. These T cells secrete high levels of IL-10 and TGF-β, significant amounts of IFN-γ and IL-5, low IL-2, and no IL-4. They also suppress T-cell responses via an IL-10- and TGF-β -dependent mechanism, and their induction can be blocked by anti-IL10R monoclonal antibody, indicating that they are Tr1 cells that are generated through autocrine production of IL-10 by iDCs. In addition, Tr1 cells induced by iDCs do not require the presence of CD4$^+$CD25$^+$ Tregs for their generation and do not express high constitutive levels of CD25 or the transcription factor Foxp3 (40). It has also been shown that a single injection of iDCs pulsed with influenza matrix peptide (MP) in healthy subjects is sufficient to induce antigen-specific IL-10-producing CD8$^+$ T cells *in vivo* and to concomitantly reduce the number of MP-specific IFN-γ-producing CD8$^+$ effector T cells (102).

Induction of Tr1 cells by tolerogenic myeloid DCs

A large body of evidence suggests that not only iDCs but also specialized subsets of tolerogenic DCs may prime Tr1 cells (2, 104–108). Tolerogenic DCs can be induced either by biological or by pharmacological agents (109, 110). For example, DCs treated with immunomodulatory cytokines such as IL-10 (111, 112), TGF-β (113), IFN-α (114), or TNF-α (115) become tolerogenic and induce the differentiation of Tregs *in vitro*. We recently demonstrated that DCs generated in the presence of exogenous IL-10 (IL-10 DCs) secrete significantly higher levels of IL-10 compared with iDCs, whereas the amounts of IL-12 are low and comparable to those produced by iDCs. Importantly, this ratio of cytokine production by IL-10 DCs is maintained upon activation with lipopolysaccharide and IFN-γ. IL-10 DCs are more powerful than iDCs in inducing Tr1 cells, because they reproducibly induce anergic T-cell lines with strong suppressive activity after only one round of stimulation (Gregori *et al.*, manuscript in preparation) (Fig. 2B).

Certain pathogens or allergens, such as *Bordetella pertussis* (116), *Lactobacillus reuteri*, *L. casei* (117), and ferruginol (118), evade immunity by priming DCs to become tolerogenic DCs, which induce Tr1 cells. Similar to infectious agents, several studies report that DCs loaded with tumor antigens can also drive the differentiation of Tr1 cells. For example, exposure of DCs to myeloma cells (119), to cyclo-oxygenase-2-over-expressing glioma cells (120), or to an adenoviral vector expressing a prostate-specific antigen (PSA) (121) can prime DCs to produce IL-10 but not IL-12p70. These latter DCs induce the differentiation of Tr1 cells, which inhibit effector T cells specific for PSA. DCs can also be modulated with endogenous proteins, such as heavy chain ferritin (122), or be engineered to express tolerogenic molecules, such as IL-10, TGF-β, CTLA-4 (123), or Serrate (a ligand for Notch proteins) (124), and ultimately drive the differentiation of Tregs that mediate antigen-specific tolerance (*Table* 2). Finally, DCs treated with immunosuppressive drugs, such as vitamin D3 and/or dexamethasone, which also modulate DC maturation, prime T cells to become anergic and suppressive (125, 126).

Molecules expressed by tolerogenic myeloid DCs involved in Treg induction

Surprisingly, the capacity of tolerogenic DCs to induce Tr1 cells does not correlate with reduced expression of canonical costimulatory molecules. Monocyte-derived DCs treated with heavy chain ferritin express high levels of the costimulatory molecules CD86 and programmed death ligand-1 and nevertheless induce the differentiation of IL-10-producing T cells (122). Similarly, *B. pertussis*-treated DCs, which induce Tr1 cells, express levels of CD40 and CD86 comparable to those of mature DCs (116). It is possible that other members of the B7/CD28 family, such as inducible costimulator (ICOS)/ICOS ligand (ICOS-L) can act as negative signaling molecules that ultimately induce the generation of Tr1 cells (127). Indeed, mature pulmonary DCs in the bronchial lymph nodes of mice exposed to respiratory allergens induce the development of IL-10-producing $CD4^+$ T cells, which inhibit allergen-induced airway hyper-reactivity via the ICOS/ICOS-L pathway (19). It also has been demonstrated that DCs expressing high levels of the immunoglobulin-like transcript 3 (ILT3) and ILT4 induce T-cell anergy (128) and promote $CD4^+CD25^+$ Treg differentiation (129). Interestingly, IL-10, IFN-α, and vitamin D3 induce upregulation of ILT3 and ILT4 on DCs, which become tolerogenic (129).

In addition to the possible role of costimulatory molecules, expression of enzymes such as indoleamine 2,3-dioxygenase (IDO) and heme-oxigenase-1 (HO-1) may also be involved in induction of Tr1 cells by tolerogenic DCs (130–132). Indoleamine 2,3-dioxygenase degrades tryptophan, an essential amino acid, and its expression is associated with tolerance induction. Human DCs expressing IDO block T-cell proliferation *in vitro* (133) and *in vivo* (134). Furthermore, induction of IDO can be enhanced by crosslinking of CD80/CD86 with CTLA-4 expressed by Tregs, thereby amplifying the regulatory network (132, 135–137). In DCs, IDO might function as a downstream mediator of the tolerogenic effects of CTLA-4 (135), possibly representing a major mechanism for the *de novo* induction of adaptive Tregs.

A new candidate enzyme involved in tolerance induction is HO-1, which degrades heme (138) and is involved in tissue protection and modulation of inflammation. HO-1 is expressed by human and rat DCs, and its expression is down-regulated during maturation. Upregulation of HO-1 expression renders DCs refractory to maturation and inhibits their capacity to induce allogeneic responses yet preserves IL-10 production (139). Importantly, overexpression of HO-1, by intragraft or systemic delivery of adenoviral vectors encoding the HO-1 gene, promotes long-term cardiac allograft survival in recipient mice and is associated with inhibition of the alloantigen-specific immune responses (140). Heme-oxigenase-1, expression of which in APCs is induced by IL-10 both *in vitro* and *in vivo*, also plays a central role in modulating protection against lipopolysaccharide-induced septic shock in mice (141). Finally, delivery of IL-10 by adenoviral vectors delays recurrence of diabetes in non-obese diabetic (NOD) mice through induction of HO-1 (142). Together, these data suggest that there is a link between IL-10 and HO-1, but the role of this enzyme in regulating Tr1 cell induction by DCs remains to be defined.

Other tolerogenic DCs and Tr1 cell induction

Not only myeloid DCs but also other subsets of DCs, including plasmacytoid DCs, can induce the differentiation of Tregs. Plasmacytoid DCs polarize T cells towards IL-10 production and regulatory activity *in vitro* (30, 143, 144). Interestingly, activated plasmacytoid DCs prime naïve $CD8^+$ T cells to become $CD8^+$ Tr1-like cells, which display poor secondary proliferative and cytolytic responses and mediate suppression through IL-10 (30). In addition, repetitive stimulation of naïve $CD4^+$ T cells with murine $CD8\alpha^+$ plasmacytoid DCs isolated from mesenteric lymph nodes results in the generation of a population of Tr1-like cells with regulatory properties (145). It has also been demonstrated that IL-10 drives the *in vitro* differentiation of a specific subset of murine DCs

characterized by high expression of CD45RB. Interestingly, $CD45RB^{high}$ DCs can also be isolated from the spleen and lymph nodes of normal mice. These naturally occurring or *in vitro*-induced $CD45RB^{high}$ DCs display a plasmacytoid-like immature phenotype, secrete high levels of IL-10 upon activation, and induce the differentiation of antigen-specific Tr1 cells both *in vivo* and *in vitro* (146).

In conclusion, iDCs can maintain homeostasis through induction of Tr1 cells in the absence of inflammation, whereas during an ongoing immune response, tolerogenic myeloid and/or plasmacytoid DCs are required. The Tregs induced by mature tolerogenic DCs become fully functional only after repetitive antigen stimulation and therefore can have a role in dampening the immune response and limiting tissue damage.

Induction of Tr1 cells *in vivo*

In vivo induction of antigen-specific Tregs to restore tolerance is an innovative and potentially powerful approach to cure a variety of immune-mediated diseases. We and others have recently shown that Tr1 cells specific for alloantigens can be induced *in vivo* in models of pancreatic islet transplantation (147, 148). Administration of rapamycin and IL-10 to diabetic mice transplanted with allogeneic pancreatic islets not only blocks acute allograft rejection but also leads to active long-term tolerance via induction of alloantigen-specific Tr1 cells (148). Interestingly, administration of IL-10 alone does not protect from allograft rejection, and it is not sufficient to induce tolerance. Although IL-10 administration reduces inflammation and generates Tr1 cells in this model, it seems insufficient to counteract the expansion and function of effector T cells. Addition of rapamycin is required for induction of operational tolerance in this model. Rapamycin blocks IL-2-induced expansion of effector T cells, without preventing Tr1 cell induction *via* TCR stimulation in the presence of IL-10. These results indicate that in addition to the generation of alloantigen-specific Tr1 cells, downmodulation of effector T cells is required to induce long-term tolerance (*Fig.* 3).

Asiedu *et al.* (147) recently demonstrated that peri-transplant treatment of diabetic non-human primates (NHPs) with anti-CD3 immunotoxin and deoxyspergualin induces stable rejection-free tolerance to allogeneic pancreatic islets transplant, which is associated with sustained elevation in serum IL-10 levels. In this *in vivo* regimen, anti-CD3 immunotoxin depletes effector T cells, whereas deoxyspergualin arrests the production of proinflammatory cytokines and the maturation of DCs. Interestingly, frequencies of Tr1 cells are significantly increased in peripheral blood mononuclear cells (PBMCs) of long-term tolerant NHP recipients as compared with controls. In addition to the increased number of Tr1 cells, the tolerant NHPs exhibit a nearly threefold increase in $CD4^+$ $CD25^+Foxp3^+$ Tregs (147). Similar to our own findings, these results suggest that induction of antigen-specific long-term tolerance requires the synergistic effect of drugs that downmodulate inflammation, block effector T cells, and differentiate Tr1 cells.

Anti-CD3 monoclonal antibodies were originally developed as a way of inducing immune suppression by depleting T cells (reviewed in 149). More recent studies, however, indicate

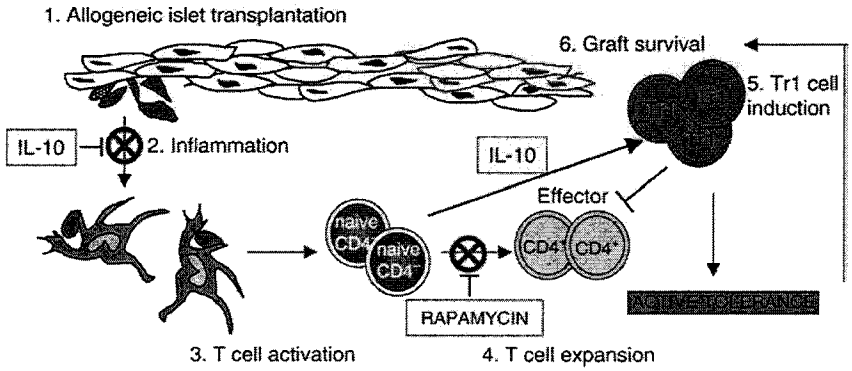

Fig. 3. Prevention of allograft rejection *in vivo* by T regulatory type 1 (Tr1) cells induced with rapamycin and interleukin-10. To prevent allograft rejection, the massive inflammation caused by the transplant must be modulated in order to reduce the recruitment and maturation of professional antigen-presenting cells. (1). Expansion of T effector cells (T_{eff}) must be tightly controlled and avoided, while conditions that permit Treg development and function should be preserved. Accordingly, treatment with rapamycin and interleukin-10 (IL-10) leads to allograft tolerance in diabetic mice transplanted with allogeneic pancreatic islets. Rapamycin and IL-10 have a general anti-inflammatory effect (2). Rapamycin, without inhibiting T-cell activation (3), blocks the early expansion of alloreactive T cells (4) and allows induction of antigen-specific Tr1 cells through IL-10 (5). With this combined therapy, graft survival and transplantation tolerance via induction of Tr1 cells is efficiently achieved (6).

that anti-CD3 monoclonal antibodies can also modulate immune responses in humans (150, 151). For example, a single course of treatment with a non-FcR binding anti-CD3 monoclonal antibody, hOKT3γ1 (Ala-Ala), leads to preservation of insulin production in patients with new-onset type 1 diabetes (150). The sustained insulin production correlates with improved glucose control and reduced use of insulin. This monoclonal antibody appears to deliver an activation signal to T cells, resulting in disproportionate production of IL-10 relative to IFN-γ *in vitro* and detectable levels of IL-10, IL-5, but rarely IFN-γ or IL-2 in the serum of treated patients. In addition, this treatment induces a population of IL-10$^+$CCR4$^+$CD4$^+$ T cells *in vivo* (152). It is therefore possible that anti-CD3 monoclonal antibodies act as inducers of Tr1 cells *in vivo*. In contrast, in murine studies, anti-CD3 monoclonal antibody treatment has been shown to induce CD4$^+$CD25$^+$ Tregs, which suppress through a TGF-β -dependent mechanism (80).

These data indicate that selected immunomodulatory compounds can induce Tr1 cells *in vivo* that mediate long-term immunological tolerance. In the future, novel pharmacological agents developed for suppression of undesired immune responses should be tested and selected based on their capacity to promote Treg function.

Role of Tr1 cells in modulating pathology in T-cell-mediated diseases

IL-10, Tr1 cells, and transplantation tolerance

The first evidence that human Tr1 cells are involved in maintaining peripheral tolerance *in vivo* came from studies in severe combined immunodeficient (SCID) patients successfully transplanted with HLA-mismatched allogeneic stem cells. Despite the HLA disparity, these patients did not develop GVHD in the absence of immunosuppressive therapy. Interestingly, high levels of IL-10 were detected in the plasma of these patients, and a significant proportion of donor-derived T cells, which were specific for the host HLA antigens and produced high levels of IL-10, could be isolated *in vitro* (26). High spontaneous IL-10 production by PBMCs before allogeneic HSC transplantation has also been associated with a subsequent low incidence of GVHD and transplant-related mortality (153, 154). More recently, high frequencies of donor T cells producing IL-10 in response to recipient alloantigens have been found to correlate with the absence of acute GVHD after allogeneic HSC transplantation in cancer patients, while low frequencies were strongly associated with severe GVHD (155). In addition, a polymorphism in the recipient's IL-10 promoter (*IL-10−592A/A* genotype), associated with high transcription levels of the IL-10 gene, has been shown to be a protective factor for severe acute GVHD and death in patients after allogeneic HSC transplantation (156). This protective effect is further enhanced by the presence of a defined polymorphism in the donor's IL-10Rβ chain (IL10RB/c238*G allele) (157). Finally, spontaneous development of tolerance to kidney or liver allograft in transplanted patients is associated with the presence of CD4$^+$ T cells that suppress naïve T-cell responses *via* production of IL-10 or TGF-β (158). Together these data indicate that Tr1 cells can regulate immune responses and induce tolerance *in vivo* in bone marrow and solid organ transplantation.

Tr1 cells regulate responses to self-antigens and autoimmunity

In healthy individuals, multiple mechanisms of central immunological tolerance eliminate or inactivate lymphocytes that bear receptors specific for auto-antigens in the thymus. Nevertheless, some autoreactive lymphocyte clones escape these mechanisms and are present within the peripheral lymphocyte pool. One way by which the pathogenic potential of these autoreactive T-cell clones is kept in check in the periphery is through Tregs. It has been suggested that the naturally occurring CD4$^+$CD25$^+$ Tregs, due to their thymic origin, are mainly involved in controlling responses to self-antigens, while Tr1 cells, which are induced in the periphery, may be important for controlling immune responses to non-self-antigens. However, there is also evidence that Tr1 cells play a central role in modulating immune responses to self-antigens. Kitani and colleagues (159) isolated self-major histocompatibility complex (MHC)-reactive Tr1 cell clones from peripheral blood of healthy individuals. These Tr1 cells inhibit proliferation of primary CD4$^+$ T cells and tetanus toxoid-specific T-cell clones via IL-10 and TGF-β. Tr1 cells specific for Desmoglein 3 (Dsg3), which is the autoantigen of *pemphigus vulgaris* (PV), were isolated from 80% of healthy carriers of PV-associated HLA class II alleles but only in 17% of PV patients (44). These Tr1 cells suppress proliferative responses of Dsg3-reactive Th cells in an antigen-specific and IL-10/TGF-β-dependent manner (44). Similarly, T cells from non-diabetic individuals carrying HLA class II molecules associated with type 1 diabetes show an IL-10 response to islet peptides, while T cells from diabetic subjects produce predominantly IFN-γ (160). Furthermore, CD4$^+$ T cells producing IL-10 but not IL-2 and IL-4 were isolated from peripheral blood and synovial tissue of rheumatoid arthritis (RA) patients, but their frequency was significantly lower compared

with that detected in control patients with non-autoimmune-mediated joint inflammation. Indeed, the frequency of these IL-10-producing $CD4^+$ Tr1 cells in the synovia of RA patients inversely correlated with the frequency of Th1 cells (161). Interferon-γ- or IL-10-producing $CD4^+$ T cells specific for the major red blood cell autoantigen, the RhD protein, were concomitantly found in the peripheral blood of patients with autoimmune hemolytic anemia (162), indicating that, although present, Tr1 cells can not fully inhibit the development of autoimmune disease (*Table* 2). These findings clearly demonstrate that self-antigen-specific Tr1 cells are present in humans and that they may play a role in maintaining self-tolerance. It is possible that skewing of self-reactive T cells toward a Treg or Th1 phenotype defines the balance between homeostasis and autoimmunity, respectively, and that unbalance in favor of Th1 cells results in pathology.

The relationship and respective role of naturally occurring $CD4^+CD25^+$ Treg and Tr1 cell subsets in regulating immune responses to self-antigens has not been completely elucidated. We demonstrated that a combined treatment with rapamycin and IL-10 blocks type 1 diabetes development in NOD mice through induction of both $CD4^+CD25^+$ Treg and Tr1 cells. Rapamycin expands the naturally occurring Tregs in the pancreas, while IL-10 promotes the differentiation of Tr1 cells in the spleen. These two Treg subsets may act through different mechanisms: $CD4^+CD25^+$ Tregs block T-cell proliferation of pancreatic autoaggressive T cells, while Tr1 cells block proliferation and migration of effector T cells to the target organ (163). It is therefore possible that both natural Tregs and induced self-antigen-specific Tr1 cells are required to restore and maintain long-term self-tolerance.

Tr1 cells regulate responses to allergens and mucosal immunity

Tr1 cells are also important in downregulating immune responses toward allergens such as nickel (48), insect venom (164), and cat allergens (165) (*Table* 2). Studies on the immune responses to non-pathogenic environmental antigens demonstrated that Tr1 cells consistently represent the dominant subset specific for common environmental allergens in healthy individuals (166). Therapies shown to be beneficial for the treatment of allergy and asthma, such as glucocorticoids, have been shown to increase IL-10 secretion by T cells (167). Furthermore, the clinical efficacy of glucocorticoids seems to be linked to the ability of T cells to produce IL-10. $CD4^+$ T cells from glucocorticoid-resistant patients fail to produce IL-10 upon *in vitro* stimulation in the presence of dexamethasone (167). Recently, it has been demonstrated that allergen-specific Tr1 cells induced *in vitro* with vitamin D3 and dexamethasone potently inhibit allergen-induced Th2 cytokine production in an IL-10-dependent manner (168). Furthermore, administration of vitamin D3 to glucocorticoid-resistant patients enhanced T-cell responsiveness to dexamethasone with an increase in IL-10 production. Collectively, these results suggest that allergen-specific Tr1 cells are important to maintain tolerance in healthy individuals and that an imbalance between allergen-specific Tr1 cells and Th2 cells may be a major factor in the development of allergy.

The mucosal immune system has an important role in maintaining the balance between protection against pathogens and unresponsiveness to both non-pathogenic resident bacteria and potentially immunogenic food antigens. It has been proposed that there are specific features of mucosal tissues that favor induction of oral tolerance through Tregs. The mucosal milieu, with its high concentrations of anti-inflammatory cytokines such as TGF-β, IL-4, and IL-10, provides a tolerogenic environment. Intestinal epithelial cells express MHC class II molecules and can process and present antigen to primed $CD4^+$ T cells. However, intestinal epithelial cells are not professional APCs, and therefore antigen presentation by these cells might lead to the development of Tregs (169). Much of the insight into the role of Tr1 cells in intestinal homeostasis has been gained from studies using the T-cell transfer model of colitis. In this model, transfer of predominantly naïve $CD4^+CD45RB^{high}$ T cells isolated from normal mice into SCID mice leads to the development of progressive inflammatory bowel disease (IBD) mediated by Th1 cells (170). Cotransfer of $CD4^+CD45RB^{low}$ T cells together with $CD4^+CD45RB^{high}$ T cells prevents the pathology due to the IL-10- and TGF-β-dependent activity of Tregs within this population (171). Furthermore, Tr1 cell clones can also prevent the development of $CD45RB^{high}$-mediated IBD when co-transferred *in vivo* (14).

Papadakis and colleagues (37) demonstrated that circulating $CCR9^+CD4^+$ T cells from normal individuals can have characteristics of mucosal T cells, i.e. they display an activated phenotype, proliferate in response to anti-CD2, have either a Th1 or Tr1 cytokine production profile, and provide support for immunoglobulin production by cocultured B cells. These cells may play an important role in small bowel immunity (172) and IBD, as well as in Crohn's and celiac diseases (37). It is tempting to speculate that $CCR9^+CD4^+$ Th1 and Tr1 cells are continuously generated in the small intestinal mucosal immune system and play an important role in effector and regulatory functions, respectively. We also recently isolated

Tr1 cell clones specific for gliadin, the immunogenic element of gluten, from the intestinal mucosa of celiac patients in remission (i.e. under gluten-free diet). These Tr1 cell clones are anergic, produce IL-10 and TGF-β , and have a strong inhibitory capacity on gliadin-specific T-cell responses *in vitro* (reviewed in 108, Gianfrani *et al.*, manuscript submitted). These data clearly indicate the involvement of Tr1 cells in suppressing immune responses towards non-harmful foreign antigens, such as allergens and food antigens, to which our organism is daily exposed (*Table* 2).

Role of Tr1 cells in disease progression

Downmodulation of the immune system by Tr1 cells can have detrimental effects in diseases controlled by the adaptive immune response. Chronic antigen stimulation can facilitate induction of Tr1 cells in an attempt to limit tissue damage by excessive immune responses. This mechanism is particularly relevant in the setting of chronic infectious diseases and cancer.

Tr1 cells in infectious diseases

A common phenomenon among organisms that cause chronic inflammation and/or persistent infections is the evolution of strategies to promote Treg development as a mechanism of immune evasion (173, 174). Although suppression of the immune response is certainly beneficial for the infectious agent, there is also clear evidence of potential benefit for the host, as Tregs limit overwhelming inflammation and ultimately preserve a source of antigen to ensure long-lived memory responses (175). One interpretation of the interplay between pathogenic organisms and Tregs is that pathogens have evolved to take advantage of the inherent regulatory mechanisms in the body, hijacking an existing system in order to evade host immune responses. Whether this regulatory response is ultimately beneficial or detrimental to the host strictly depends on the type of infection.

Multiple different types of Tregs have been implicated in immune responses to infections, including both Tr1 and $CD4^+CD25^+$ Tregs. The phenomenon of infectious tolerance, the difficulty in identifying sources of cytokines *in vivo*, and the inadequacy of CD25 as a maker of a Treg subset have often made it difficult to define distinct roles for these two subsets in mouse models. We consider data indicating a role for IL-10 in suppression of immune responses as strong evidence for the presence of Tr1 cells. Treatment of mice with a killed *Mycobacterium vaccae*-suspension results in the generation of antigen-specific $CD4^+CD45RB^{low}$ Tr1 cells, which confer protection against airway inflammation. This antigen-specific inhibition is mediated by IL-10 and TGF-β (176). Pathogen-specific Tr1 cells are also induced at the mucosal surface during *B. pertussis* infection, where local Th1 cell-mediated responses are suppressed. T regulatory type 1 cell clones specific for filamentous hemagglutinin (FHA) and pertactin can be generated from the lungs of *B. pertussis*-infected mice (*Table* 1). These Tr1 cell clones secrete high levels of IL-10 but not IL-4 or IFN-γ and suppress immune responses against *B. pertussis*. Furthermore, FHA inhibits IL-12 production by DCs, whereas production of IL-10 by DCs, which direct differentiation of naïve T cells into Tr1 cell subtype, is enhanced (116). Mice immunized with cholera toxin in the presence of antigen give rise to antigen-specific Tr1 cells (177, 178) (*Tables* 1 and 2). Moreover, *Leishmania major* (175), hepatitis C (179), filarial nematodes (180), and *Helicobacter hepaticus* have been shown to induce IL-10 secretion by APCs as well as the differentiation of Tr1 cells *in vivo* (181) (*Table* 1). This phenomenon is not only relevant for harmful persistent pathogens but also for the regulation of immunity to commensal organisms. Evidence for the requirement of IL-10 and Tregs in preventing inappropriate mucosal inflammation is substantial and has been extensively reviewed by Powrie *et al.* (182).

A general trend is that persistent infectious organisms have evolved sophisticated mechanisms to induce IL-10 production, either by inducing host APCs and/or T cells to produce this cytokine or by encoding their own IL-10 homologues. Regardless of the mechanism, the resulting IL-10-rich environment promotes tolerogenic APCs and the differentiation of pathogen-specific Tr1 cells. For example, activation of CD46 on T cells by a variety of viral and bacterial pathogens can directly enhance IL-10 production by T cells and differentiation into Tr1 cells (49, 183). Alternatively, as discussed above, pathogen-derived molecules, such as FHA (116) or adenylate cyclase toxin (184), can act indirectly at the level of DCs to suppress IL-12 production and thereby enhance APC-derived IL-10 production. Examples of pathogens encoding suppressive IL-10 homologues themselves include human cytomegalovirus and Epstein–Barr virus (EBV) (9, 10, 185). The Tr1-inducing capacity of EBV in particular has been extensively studied (185, 186), and there is strong evidence that this phenomenon not only promotes viral persistence but also may contribute to immune suppression in Hodgkin's lymphoma (186).

There is growing evidence that a key step in the process of pathogen-specific Treg development involves their capacity to traffic to sites of inflammation and respond directly to a variety of innate stimuli. For example, $CD4^+CD25^+$ Tregs express a subset of Toll-like receptors (TLRs), and TLR ligands

can enhance their suppressive function (187, 188). To date, there are no reports that document the presence or absence of TLRs on Tr1 cells. However, there is evidence suggesting that pattern recognition receptors may modulate their function both directly and indirectly via their capacity to regulate IL-10 production by other cells. For example, stimulation of DCs with peptidoglycan (which binds to NOD1) or β-glucan (which binds to dectin-1) increases IL-10 production (185, 190). In addition, TLR4-deficient mice display a decrease in IL-10 production in response to *B. pertussis* infection, do not develop Tr1 cells, and have an increase in inflammatory pathology (191). Future work will be required to clarify whether Tr1 cells express a unique subset of TLRs or whether these effects are strictly mediated indirectly, via regulation of cytokine production from APCs. Thus, the concept that many innate stimuli may contribute to the development of pathogen-specific Tr1 cells warrants further investigation.

Tr1 cells in cancer

The immune system plays a crucial role in preventing the outgrowth of neoplastic cells, both at disease onset and during metastatic transformation. It is well-known that defective immune responses associated with primary immunodeficiencies (192) or long-term immunosuppression in patients receiving allogeneic tissue transplants (193, 194) result in an increased risk for the development of cancer. In addition, tumor cells evolve mechanisms to evade the immune responses, for example by downregulating their own expression of HLA class I molecules and thereby escaping recognition by cytotoxic T cells (CTLs) (195, 197).

The capacity of IL-10 to downregulate immune responses has prompted intensive investigation into its role in the pathogenesis and progression of cancer. Elevated levels of serum IL-10 correlate with different types of tumors as well as with unfavorable prognosis, especially for B-cell neoplasias such as lymphoma (reviewed in 8). In contrast, expression of IL-10 by chronic lymphatic leukemia (CLL) has been associated with a favorable outcome (198). Furthermore, transgenic mice constitutively expressing high levels of IL-10 are not impaired in their ability to clear allogeneic tumors (199), consistent with the observation that IL-10 has stimulatory effects on the cytotoxic and proliferative capacity of antigen-specific $CD8^+$ effector T cells (8–11).

Considerable efforts have been made to elucidate the role of Tregs in downmodulating tumor-specific immune responses. Most studies have focused on the role of $CD4^+CD25^+$ Tregs, due to the relative ease of identification by cell-surface staining and elimination in monoclonal antibody-based depletion studies. Evidence for the role of $CD4^+CD25^+$ Tregs in suppressing anti-tumor immune responses in a variety of mouse models is compelling (200–202). However, most of these Tregs mediate their suppressive activity in a cytokine-dependent manner. $CD4^+CD25^+$ Tregs have been shown to infiltrate murine fibrosarcoma *in vivo*, mediating local suppression of $CD8^+$ effector T cells at the tumor site in an IL-10 and TGF-β-dependent fashion (203). $CD4^+CD25^+$ Tregs also suppress T-cell-mediated immune responses against a murine colon carcinoma via a TGF-β-dependent mechanism (204). However, $CD4^+CD25^+$ Tregs have also been shown to be able to inhibit the outgrowth of murine colon carcinoma in an IL-10-dependent mechanism, possibly via decreasing inflammatory responses at the intestinal mucosa and/or induction of apoptosis in tumor cells through downregulation of cyclo-oxygenase-2 (205, 206).

In humans, $CD4^+CD25^+$ Tregs are found in the ascites and tumor infiltrating lymphocytes (TILs) of ovarian carcinoma patients, and their presence has been associated with poor prognosis (207). Migration of Tregs appears to be preferentially directed towards ascites and tumor tissues and not to draining lymph nodes, and this migration could be stimulated by tumor cell and microenvironmental macrophage-derived CCL22 (203). Increased numbers of $CD4^+CD25^+$ Tregs are found in the peripheral blood of patients affected by different types of neoplastic diseases, including breast and pancreas carcinoma, as compared with healthy controls (208). $CD4^+CD25^+$ Tregs can represent up to 20% of lymphocytes infiltrating the tumor or the draining lymph nodes of these patients, and they have potent suppressive activity when co-cultured with anti-CD3 activated $CD8^+$ or $CD4^+$ effector T cells (208). Finally, $CD4^+CD25^+Foxp3^+$ Tregs specific for the tumor antigen LAGE-1 were isolated from TILs, providing further evidence that tumor antigen-specific responses can be hampered by induction of tumor antigen-specific Tregs (209).

Based on these data, it has been hypothesized that successful immunotherapy must simultaneously stimulate the effector T cells mediating an anti-tumor response and reduce or block Treg function. In a recent study, IL-2 was found to significantly expand $CD4^+CD25^+$ Tregs in the peripheral blood of melanoma and renal cancer patients, providing a possible rationale for the low (15–20%) response rate to IL-2 therapy in these patients (210). High frequencies of $CD4^+CD25^+$ Tregs in the peripheral blood are correlated with poor prognosis in patients affected by CLL, and the frequency and suppressive function of these cells is reduced by treatment with cyclophosphamide and fludarabin (211). Thus, reduction of immunosuppression by Tregs might be

one of the mechanisms underlying the therapeutic benefit of these chemotherapeutic agents.

The association between Tr1 cells and immunological tolerance to tumors is currently poorly understood. Marshall and coworkers (186) showed that $CD4^+$ T lymphocytes infiltrating lymph nodes of Hodgkin's lymphoma patients are enriched in both Tr1 and $CD4^+CD25^+$ Tregs and that their suppressive function is mediated via a mechanism that depends on both IL-10 and CTLA-4. Seo and collaborators (212) have shown that Tr1 and Th3 regulatory T cells can be isolated from TILs in the B16 melanoma model and that adoptive transfer of these cells facilitates tumor growth in syngeneic animals. Importantly, the suppressive activity of the Tregs was mediated by TGF-β and IL-10, and injection of IL-10 into the tumor site markedly promoted tumor progression. In contrast, Segal *et al.* (213) provided evidence for a protective role of IL-10 in terms of tumor outgrowth in a murine glioma model, possibly by enhancing CTL or NK cell function.

Overall, it is possible that different types of Tregs are recruited to and possibly also differentiated at tumor sites and that this mechanism is a major one by which tumor cells evade immunological control. This assumption indicates that the success of clinical protocols for cancer immunotherapy is dependent on the capacity of these protocols not only to boost anti-tumor responses but also to downregulate Treg function.

Immunodeficiencies due to defects in Tregs

Mutations in genes that play a role in establishing tolerance during the early stages of T-cell development can result in immunodeficiencies characterized by early onset systemic autoimmunity. The development of similar autoimmune manifestations in this family of diseases, which all seem to result from the failure of different tolerance mechanisms, demonstrates that the process is not redundant and that many different molecular defects can lead to a similar clinical phenotype. For example, mutations in the AIRE (autoimmune regulator element) gene result in defective central T-cell deletion and escape of autoreactive T cells to the periphery. These cells target multiple endocrine glands giving rise to the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome, also known as APS-I (214, 215). Similar autoimmune polyendocrinopathies are also described in the APS-II syndrome. Although the genetic defect of this autoimmune disease has yet to be determined, the immunopathology could be due to defective $CD4^+CD25^+$ Tregs (216), because the clinical phenotype is very similar to that described in nude mice reconstituted with $CD4^+$ T cells depleted of $CD25^+$ T cells (56).

The clearest example of genetically determined impairment of peripheral tolerance is the pathology due to mutations in the Foxp3 gene. In the mouse, mutations in Foxp3 hinder the development of $CD4^+CD25^+$ Tregs, leading to severe autoimmunity and uncontrolled lymphoproliferation that can be rescued by transfer of wildtype $CD4^+CD25^+$ Tregs (60–62). In human, mutations in the Foxp3 gene are responsible for a severe autoimmune disease, called immune dysfunction, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, in which the autoreactive T cells target the pancreatic β islets and the intestinal mucosa, thereby causing early onset type 1 diabetes and refractory enteritis (217). Typically, eczema associated with high immunoglobulin E serum levels is also present. Interestingly, in IPEX patients, mutation of Foxp3 does not always lead to abrogation of protein expression and lack of $CD4^+CD25^+$ (218) Tregs (Bacchetta *et al.*, manuscript submitted). Nevertheless, Tregs from IPEX patients show impaired suppressor functions, accompanied by reduced IL-2 and IFN-γ production by effector T cells. Based on these data, we hypothesize that Foxp3 not only plays a role in the differentiation and function of $CD4^+CD25^+$ Tregs in humans but also participates in regulatory pathways triggered by TCR activation of effector T cells (218). Data indicating that Foxp3 functions as suppressor of cytokines by interacting with nuclear factor for activation of T cells (NFAT) and NFκB are in line with this notion (219, 220).

In these genetic autoimmune diseases, the lack of tolerance is primarily due to defective thymic development, leading to defective differentiation or function of natural Tregs. It remains to be addressed whether peripheral differentiation of adaptive Tr1 cells upon exogenous antigen exposure is also impaired in these patients. In addition, the potential role of an IL-10 deficiency has not been examined. Indeed, it is possible that IL-10-driven Tr1 cell differentiation in the periphery is not affected and that these cells could possibly modulate the autoimmune response and limit pathology in patients with a mild clinical phenotype. Recently established *in vitro* and *in vivo* methods to induce adaptive antigen-specific Tregs could therefore be exploited to design new therapeutic approaches for these rare but severe genetic autoimmune diseases.

Cellular therapy with human Tregs

Allogeneic HSC transplantation is the treatment of choice for a variety of disorders of the hematopoietic system including hematologic malignancies, such as leukemia, lymphoma, and multiple myeloma, and genetic diseases, such as β -thalassemia and primary immunodeficiencies (221). Donor-derived T cells administered along with the graft or separately as donor lymphocyte infusions (DLIs) are crucial for the

clinical success of allogeneic HSC transplantation, since they accelerate immune reconstitution and eliminate residual neoplastic cells [graft-vs.-leukemia activity (GVL) and graft-vs.-tumor activity (GVT)]. Unfortunately, the same T cells mediating GVL/GVT can also cause acute and chronic GVHD, one of the main clinical complications after allogeneic HSC transplantation. In fact, the target antigens of GVL/GVT and GVHD are at least partially overlapping, and in support of this notion, it has been shown that the two clinical phenomena are frequently associated (222, 223). Considerable effort has been made over the recent years to obtain a GVL effect without GVHD. The current approaches include the use of T lymphocytes enriched for GVL target antigens by *in vitro* stimulation with tumor cells (224) and the use of donor T lymphocytes engineered to express a drug-inducible suicide gene that allows their rapid elimination through administration of the relevant drug (225). The limitations of these approaches are related to the narrowing of the repertoire of T cells in the DLIs, which reduces the extent to which immune reconstitution is obtained, as well as to safety issues regarding the use of genetically modified cells.

A promising alternative approach to tackle this problem involves the use of Tregs as a cellular therapy after allogeneic HSC transplantation. Due to the relative simplicity of obtaining large numbers of purified $CD4^+CD25^+$ Tregs by selection for high levels of CD25 expression (89, 90, 226), the potential use of natural Tregs has been extensively explored in preclinical murine models of cellular therapy (88). In an elegant study by Edinger and colleagues (227), $CD4^+CD25^+$ Tregs were shown to prevent acute GVHD by suppressing alloreactive T-cell expansion without impairing the anti-tumor effector functions mediated by these cells. In a murine model of HSC transplantation into fully allogeneic recipients followed by engraftment of leukemia cells, it was shown that a 10-fold lower limiting threshold number of effector T cells is required for protective GVL as compared with lethal GVHD. In this model, $CD4^+CD25^+$ Tregs inhibit the expansion but not the functions of effector T cells. Thus, the low number of effector T cells present was below the threshold for the induction of GVHD but sufficient to maintain GVL. Other authors showed that the capability of $CD4^+CD25^+$ Tregs to protect mice from acute GVHD *in vivo* is critically dependent on expression of the lymphocyte-homing receptor CD62L (227) and the chemokine receptor CCR5 on Tregs, which promote their homing to liver, lung, spleen, and mesenteric lymph nodes (228).

Interleukin-10 and TGF-β have been shown to induce murine anergic T cells that suppress alloreactive T-cell responses *in vitro* and significantly reduce GVHD lethality *in vivo* after injection of $CD4^+$ effector T cells into HLA class II-disparate recipients (94, 95). Furthermore, $CD4^+$ T cells obtained from allogeneic MLR in the presence of IL-10 reduce skin graft GVHD in skin explant assays (220). Using a donor-recipient MLR system in the presence of exogenous IL-10, we established a protocol for induction and expansion of donor-derived alloantigen-specific anergic T cells (230) (Fig. 2*A*). We demonstrated that donor PBMCs cultured *in vitro* with irradiated host PBMCs in the presence of IL-10 for 10 days become anergic towards the host antigens, while they preserve the ability to proliferate in response to third party and nominal antigens. Importantly, these IL-10-anergized T cells are highly enriched for alloantigen-specific Tr1 cell precursors (230) (Fig. 2*A*). Based on these results, we have recently implemented the first protocol of cellular therapy with Tr1 cells after HLA-haploidentical HSC transplantation (http://www.risetfp6.org).

Human leukocyte antigen-haploidentical transplantation offers a valuable source of HSCs to most patients in need of a bone marrow transplant when matched donors are unavailable. A megadose of highly purified $CD34^+$ HSCs depleted of mature T cells is crucial for promoting engraftment without GVHD. However, T-cell-depleted transplants are at high risk of recurrent life-threatening infections because of the lack of prompt immune reconstitution and the long-lasting immunodeficiency. In our clinical trial, donor T cells anergized *in vitro* in the presence of IL-10 are infused post-transplant into the host with the ultimate goal to provide immune reconstitution with donor T cells that are anergic towards host antigens and contain precursors of host-specific Tr1 cells. The administered cells should therefore include alloantigen-specific T cells with the ability to differentiate into fully competent Tr1 cells, and also T cells able to respond to infectious agents and presumably to provide a GVL effect. Six patients transplanted from haploidentical family donors for high risk hematologic malignancies have been treated with this cellular therapy (M. G. Roncarolo, unpublished data). In this cohort of patients, no toxicity and no cases of drug-resistant acute GVHD have been observed. This approach, which is currently being tested in the context of HLA-haploidentical HSC transplantation, has the potential to be applied to unrelated bone marrow transplants and to solid organ transplantations. Cellular therapy with $CD4^+CD95^+$ Tregs and/or Tr1 cells to modulate pathology and restore tolerance in other T-cell mediated diseases, including Type 1 diabetes systemic lupus erythematosus, allergy, and in organ transplantation should be considered.

Future perspectives

Our increasing knowledge of the biology and mode of action of Tregs has changed our view of the pathophysiological

mechanisms underlying a variety of T-cell-mediated disorders and has opened new possibilities for the cure of these diseases. Thanks to the increasing interest in this field and to the work of several investigators, including our own group, much progress has been made over the last 5 years in understanding these subsets of T cells with regulatory function. It has been established that Tr1 cells and $CD4^+CD25^+$ Tregs are two distinct populations of Tregs with different biological properties, including different mechanisms of actions and responses to cytokines. It has become clear that Tr1 cells are inducible in an antigen-specific fashion and can therefore be defined as adaptive Tregs, whereas $CD4^+CD25^+$ Tregs are primarily naturally occurring cells that are selected in the thymus, are probably specific for self-antigens, and probably represent a separate T-cell lineage. We have established different methods to induce Tr1 cells *in vitro* and *in vivo* in different experimental systems, and we have shown that IL-10 is always the key factor in the induction of Tr1 cells. Furthermore, we have understood that depending on the mode of induction, Tr1 cells can slightly vary in their cytokine production profile but that IL-10 production in the absence of IL-4 is the consistent hallmark of these cells. We have also determined that Foxp3 is a marker for natural $CD4^+CD25^+$ Tregs but not for adaptive Tr1 cells, and in the human system, this transcription factor may have an additional role beyond its function in the development of $CD4^+CD25^+$ Tregs.

Many of the questions which were set forward more than a decade ago at the beginning of our work in this field have been answered (231, 232). However, the large body of information provided by *in vitro* studies and by studies dissecting the role of Tregs in different disease models and in different human pathologies has also generated new questions and some degree of confusion. Results from *in vivo* studies have not always been consistent with *in vitro* observations. Furthermore, many contradictory results by different groups have been reported. This disparity is due not only to the lack of specific markers for Tr1 cells and to the inadequacy of CD25 as a specific marker for natural Tregs but also to the undefined mechanism of suppression by $CD4^+CD25^+$ Tregs and the paucity of studies in which Tregs were characterized at the clonal level. In addition, the antigen specificity of these cells was determined on a very limited scale. Overall, these factors have significantly contributed to the generation of conflicting and sometimes confusing results, which consequently have led to an increasing amount of skepticism on the relevance of these cells.

It is crucial that over the coming years better standardized and reproducible approaches are used to define and characterize Tregs at the cellular and molecular level. The recent advances in the areas of proteomics and genomics will provide valuable tools for this purpose. To advance this field and fully understand the functional role of the different Tregs, the identification and use of specific Treg markers and/or molecular signatures is absolutely required. Only by using these approaches will we be able to address the questions that still remain open, including the relationship between $CD4^+CD25^+$ Tregs and Tr1 cells and their respective roles in modulating the immune response and in maintaining tolerance. In addition, the signaling pathways involved in the regulatory activity of Tr1 cells and, importantly, the homing receptors governing their trafficking *in vivo* need to be characterized. Finally, the identification of specific markers will enable us to rapidly purify Tr1 cells *ex vivo* and to follow their fate *in vivo*. Results from these studies will not only contribute to the advancement of the field but also allow us to fully exploit the therapeutic potential of Tregs. Hopefully, in the coming years we will witness the design and implementation of clinical protocols based on the *in vivo* induction of Tregs or, alternatively, cellular therapy with *in vitro*-generated Tregs to cure autoimmune diseases, to prevent GVHD and organ rejection, and to avoid immune responses to the products of therapeutic transgenes. Such clinical trials will be critical to convincingly prove the fundamental role of Tregs in modulating the immune response and inducing immunological tolerance *in vivo*.

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